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Yolk carotenoids have sex-dependent effects on redox status and influence the resolution of growth trade-offs in yellow-legged gull chicks

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Avian eggs are rich in carotenoids, which derive from maternal diet where they may be available in limiting amounts. Egg carotenoids may accomplish major roles in antioxidant protection or modulate physiological functions and growth, interfering with offspring redox status, potentially in a sex-dependent way. In this study of maternal effects in relation to sex and laying order of yellow-legged gull *(Larus michahellis)* chicks, we analyzed the consequences of increased yolk lutein concentration on plasma antioxidant capacity (AOC) and an index of early oxidative damage (reactive oxygen metabolites, ROM), till 9 days after hatching. To this end, for the first time we directly manipulated yolk lutein, thus avoiding any effect on other components of egg quality due to maternal supplementation before laying. Lutein did not increase AOC but increased ROM in males and in first-laid chicks. Hence, lutein did not act as an antioxidant and determined increased early oxidative damage, possibly because of upregulation of immune or other physiological functions, but these effects were sex-related and apparent in first-laid chicks with larger yolk lutein supply. ROM positively covaried with AOC, suggesting a trade-off between AOC and oxidative damage. Moreover, lutein injection altered the covariation between body size or immunity and AOC or ROM. Carotenoids may thus not be major antioxidants in birds and rather affect redox status by increasing oxidative damage in a sex-dependent way and interfere with the resolution of growth trade-offs. In the absence of sex-related allocation, maternal decisions on egg carotenoid concentration may depend on the balance between divergent effects on either sex. *Key words:* antioxidants, carotenoids, egg, laying order, maternal effects, oxidative stress, sex. *[Behav Ecol]*

Parents contribute more than their genome to the offspring (Jablonka and Lamb 1995; Badyaev 2008). Parental phenotype and environmental conditions shape the pre- and postnatal ecological milieu of the progeny, ultimately translating into offspring phenotypic variation (Mousseau and Fox 1998a, 1998b). Such "maternal effects" not only mediate the downstream transmission of physiological and environmental influences to the next generation, with consequences for mean offspring phenotype (Badyaev and Uller 2009). They can also adaptively modulate phenotypic variation among offspring according to their genetic makeup (e.g., sex) and reproductive value as influenced by birth order and social environment (Mousseau and Fox 1998a; Badyaev 2002; Badyaev et al. 2005; Saino et al. 2010). Maternal effects can thus alter the covariance between genotype and phenotype and evolve as epigenetic influences on offspring, functioning to promote parental fitness (Wade 1998; Wolf et al. 1998).

The extent to which maternal effects contribute to reproductive strategies depends on several conditions. Adaptive maternal effects are more likely to be effective when rearing conditions vary predictably, as in "structured" broods (Hall et al. 2010) where younger siblings face harsher conditions (Schwabl 1996; Eising et al. 2001; Groothuis et al. 2006). Second, mechanisms must exist that allow tuning of maternal effects according to sensitivity and need as influenced, for example, by sex (Badyaev et al. 2006). Moreover, maternal effects can be constrained by trade-offs between maternal investment and self-maintenance (Zera and Harshman 2001).

Maternal endogenous (e.g., hormones) or dietary (e.g., antioxidants) egg components regulate developmental processes (Surai 2002; Groothuis et al. 2005). Eggs must thus be adequately equipped with these maternal effects to ensure homeostatic development and protection from the consequences of metabolic activity associated with growth. Indeed, normal metabolic and immunological processes entail the production of reactive oxygen species (ROS) (Halliwell and Gutteridge 2007). When the production of ROS exceeds the antioxidant capacity (AOC), damage to DNA, lipids, and proteins or activation of cellular signaling involved in pathogenesis may ensue (Balaban et al. 2005; Costantini 2008; Monaghan et al. 2009). The imbalance between ROS and antioxidant defense (oxidative stress) can impair viability and other fitness components (Beckman and Ames 1998; Finkel and Holbrook 2000). Intense metabolism during embryonic and postnatal development causes massive ROS production (Alonso-Alvarez et al. 2007; Nussey et al. 2009), and egg substances thus play a crucial role in antioxidant defense (Surai et al. 1999, 2003; Surai 2002).

Major roles in the vertebrate antioxidant system are accomplished by endogenous enzymes and by exogenous elements and substances (e.g., selenium, tocopherols, carotenoids; Surai 2002; Monaghan et al. 2009). Much of the evolutionary ecological research on antioxidants has converged on carotenoids, which contribute to color signals involved in sexual and parent–offspring communication (Goodwin 1984; Monaghan et al. 2009), and have a broad spectrum of physiological actions. Being quenchers of singlet oxygen and scavengers of free radicals, they serve as antioxidants (Halliwell and

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Gutteridge 2007). They also contribute to regulate gene expression, cell proliferation, and cell–cell communication (Edge et al. 1997; Surai 2002; Chew and Park 2004). Moreover, they modulate immunity and concur to fend off the pathological effects of immune processes (von Schantz et al. 1999; Møller et al. 2000; Surai 2002; Koutsos et al. 2003, 2006; Saino et al. 2003; Konjufca et al. 2004).

Avian eggs are rich in carotenoids (Surai 2002; Biard et al. 2009), which play a fundamental role for embryo and postnatal growth (Surai et al. 2001; Surai 2002; McGraw et al. 2005). Egg carotenoids also have organizational effects on several traits, including absorption and utilization of carotenoids in adulthood, and thus have enduring effects on viability and on the expression of epigamic traits (Fitze et al. 2003; Koutsos et al. 2003, 2006, 2007; Isaksson et al. 2006; Biard et al. 2007). The relevance of carotenoids to avian embryonic development has been mainly attributed to their antioxidant properties (Surai 2002), but their role as antioxidants has been questioned (Hartley and Kennedy 2004; Costantini and Møller 2008; Isaksson and Andersson 2008). Although their mechanisms of action are still largely obscure, their importance during the pre- and postnatal life is well supported (Surai 2002; Saino et al. 2003; Alonso-Alvarez et al. 2004; Costantini, Cardinale, and Carere 2007).

Because their dietary availability may be limiting (Olson and Owens 1998; Blount et al. 2004), carotenoids may mediate physiological trade-offs between signaling and antioxidant defense or immunostimulation but also between parental self-maintenance and reproduction (Lozano 1994; Monaghan et al. 2009). Given their modulatory actions, they may be involved in the resolution of trade-offs among functions, such as growth or immunity, and individual redox status. Ecological limitation and sex-biased sensitivity (De Neve et al. 2008; Romano et al. 2008; Newbrey and Reed 2009) may select for differential transmission of carotenoids to eggs of either sex (Badyaev et al. 2006) or, when no sex-related allocation to the eggs evolves, for maternal strategies that maximize the benefit-to-cost balance of carotenoids effects on either sex (Romano et al. 2008). Some studies have provided evidence for a covariation between egg carotenoid concentration and sex (Verboven et al. 2005; Badyaev et al. 2006; but see Groothuis et al. 2006; Romano et al. 2008). Because position in the laying/hatching sequence affects the reproductive value of individual offspring, carotenoids may be allocated to eggs differentially according to laying order (Royle et al. 1999, 2003; Blount, Surai, Nager, et al. 2002; Saino et al. 2002; Saino, Bertacche, et al. 2008).

Several studies have investigated the effects of egg carotenoids on the offspring by supplementing laying mothers. Supplementation increases the concentration of carotenoids and resistance to lipid peroxidation in the yolk and offspring tissues up to weeks after hatching (Surai et al. 1996, 2003; Blount, Surai, Houston, and Møller 2002; Koutsos et al. 2003; Karadas et al. 2005; Ewen et al. 2006), and offspring from carotenoid supplemented mothers perform better (Koutsos et al. 2006, 2007; De Neve et al. 2008; but see Remeš et al. 2007; Ewen et al. 2009). Maternal supplementation is a valuable approach when testing for ecological limitation of carotenoids to laying performance, but it does not allow a direct test of the effect of egg carotenoids on offspring phenotype because any consequence on the offspring could be mediated by maternal physiology, and thus by allocation to the egg of other constituents, including other antioxidants (Surai et al. 2003; Ewen et al. 2006). Supplemental carotenoids may also interfere with the resolution of maternal trade-offs (Bertrand et al. 2006) or influence egg content of immune factors (Blount, Surai, Nager, et al. 2002), with consequences for offspring arising independently from egg carotenoid concentration. Conversely, manipulation of maternal substances directly in ovo (Saino et al. 2003, 2006; Tschirren et al. 2005) can disentangle such indirect effects from the direct effects of egg components.

In this study of maternal effects mediated by carotenoids in relation to sex and laying order of yellow-legged gull (*Larus michahellis*) chicks, for the first time we analyzed the effect of increased yolk carotenoid concentration on plasma AOC and oxidative damage, indexed by the concentration of reactive oxygen metabolites (ROM; see MATERIALS AND METHODS), by manipulating lutein concentration in ovo. The effects of lutein on growth and survival are reported in Romano et al. (2008). Because the importance of carotenoids as antioxidants in birds is contentious, no unequivocal predictions for the effect of lutein on chick AOC and ROM could be made. If carotenoids are important antioxidants, we predicted larger AOC in lutein chicks. Lutein could reduce ROM, if carotenoids are strong antioxidants. If, on the other hand, carotenoids enhance growth or interfere with physiological trade-offs, lutein could increase ROM because the effect of enhanced growth on ROS production was not countered by increased AOC. In the latter scenario, we also predicted a larger positive effect of lutein on ROM in males, which may be more susceptible to oxidative stress because of differences in androgen profiles (see Royle et al. 2001).

The second main aim was to investigate whether lutein influenced the covariation between growth or immune response and AOC or ROM. The expected interference of increased lutein yolk concentration depends on the specific action of carotenoids. For example, if lutein has antioxidant effects, a positive relationship between growth and ROM can be expected among control rather than lutein chicks, whereas an even steeper relationship could be expected if lutein stimulates growth with no major antioxidant effects. Given the uncertainty on the physiology of carotenoids, however, we refrain from making predictions on the effects of lutein on the resolution of trade-offs between growth or immunity and redox status.

MATERIALS AND METHODS

The yellow-legged gull is a monogamous semicolonial species with biparental care of the eggs and of the altricial offspring (Cramp 1998). Modal clutch size is 3 eggs, which are laid at 2–3 days intervals and decline in size with laying order, with third (c-) eggs being markedly smaller and second (b-) eggs being slightly smaller, on average, than their first (a-) sibling egg (Rubolini et al. 2009). Hatching of sibling eggs is asynchronous and takes on average 2 days to be completed, resulting in "structured" broods with a marked hierarchy in size and competitive ability. Males are larger than females already at hatching and either sex is differentially susceptible to maternal effects via hatching asynchrony (Saino et al., 2011).

Previous findings from the same gull population relevant to the present experiment are that the concentration of lutein, the main xanthophyll in the eggs and plasma of this species (Pérez et al. 2008; Saino, Bertacche, et al. 2008), is smaller in c- compared with a- or b-eggs (Saino, Bertacche, et al. 2008; Karadas F, Rubolini D, unpublished data). Lutein concentration does not predict yolk AOC, suggesting a minor role of lutein as a yolk antioxidant, nor AOC in the plasma of the chicks (Saino, Bertacche, et al. 2008). No differential allocation of lutein or other carotenoids to eggs of either sex was observed in two independent studies of the same population (Romano et al. 2008; Karadas F, Rubolini D, unpublished data).

Field procedures

The study was conducted in the Comacchio lagoon (NE Italy, lat 44°20′N–long 12°11′E), at a colony with more than 400

breeding pairs, during March–June 2007. We regularly visited each of the 2 parts of the colony every second day starting at the beginning of the nesting period. New nests and eggs were individually marked. Because nests were visited every second day and the newly laid eggs were treated on the same day when they were found, 2 days or less elapsed between egg laying and treatment, consisting in the injection of a physiological dose of lutein or of a control solution (see below). When a new egg was found, it was removed from the nest, temporarily replaced with another yellow-legged gull egg, and taken to a nearby building for inoculation procedures. The details of the injection procedures and tests that injected lutein actually reached the yolk are reported in Romano et al. (2008). After being injected, the eggs were then put back to their original nest within 4 h.

The decision of injecting lutein rather than another carotenoid or a mixture of the carotenoids found in yellow-legged gull eggs from the same colony was taken because: lutein is the most abundant carotenoid in the eggs of this species (Pérez et al. 2008; Saino, Bertacche, et al. 2008; Karadas F, Rubolini D, unpublished data); egg lutein concentration predicts chick phenotype more strongly than other carotenoids (Saino, Bertacche, et al. 2008); and designing injection of a mixture of carotenoids is difficult because eggs vary widely in both relative and absolute concentrations of individual carotenoids (Saino, Bertacche, et al. 2008; Karadas F, Rubolini D, unpublished data). The clutches were assigned sequentially, according to the order in which they were found, to the following treatment schemes (nest, a-, b-, c-egg): nest 1, lutein injection (L), control injection (C), L; nest 2, C-L-C; nest 3, L-C-C; nest 4, C-L-L, and so forth with the following nests.

Our aim was to increase the concentration of lutein within its natural range of variation (Romano et al. 2008). We therefore injected an amount of lutein corresponding to that needed to increase its concentration by 1 standard deviation (SD) of the concentration recorded in the egg yolk. However, in order to decide the absolute amount of lutein due to be injected in individual eggs, we had to take variation in yolk mass into account. We did so by estimating yolk mass based on a linear regression equation on total egg mass and while taking into account a positive relationship between mean egg (and thus yolk) mass and variance, as described in full details in Romano et al. (2008). This procedure ensured that lutein concentration in the yolk of each egg was increased by approximately 1 SD and, thus, that our results were obtained under a condition of physiological increase in lutein concentration. The amount of lutein injected into eggs depended on their mass: 75 μg for 66–75 g eggs, 100 μg for 76–85 g eggs, 155 μg for 86–95 g eggs, and 210 μg for 96–105 g eggs (Romano et al. 2008). Lutein (FloraGLO Lutein 20% Liquid in Safflower oil) was always diluted in 30 μl safflower oil. Control eggs were injected with 30 μl safflower oil alone (for further details, see Romano et al. 2008). Safflower oil contains little carotenoids (lutein and zeaxanthin) and the amount of safflower carotenoids we injected via 30 μl oil corresponds to $<$0.09% of the lutein dose. Because FloraGLO also contains a small amount of zeaxanthin, injection also vehiculated an amount of zeaxanthin corresponding to a maximum of 11% of the total amount of injected lutein.

All the nests where laying had been completed were visited daily starting well before the expected hatching date to check for hatching status. Chicks could be assigned to their original egg by injecting a small drop of food dye in the eggs at pipping stage (see Romano et al. 2008) and were individually marked after hatching.

For the purposes of the present study, we measured egg mass on injection and chick body mass (nearest g; expressed in g) and tarsus length (nearest 0.1 mm; expressed in mm) 1 and 9 days after hatching. At later ages, vagile gull chicks can move considerable distances from their nest and sometimes can hardly be found in the thick herbaceous vegetation. Exact age in days could be assigned to each chick at the time when they were found hatched. Because some chicks could not be found the day when measurement was planned, exact age at measurement was included as a covariate in the analyses. The mean age at measurement was 8.90 days (0.65 SD; age 8: 24.2% of the chicks, 9: 63.4%, 10: 10.2%, 11: 2.2%). At the age of 9 days, we also performed an in vivo immune test (the phytohemagglutinin, PHA, skin test), according to a standard protocol (Saino et al. 1997; Tella et al. 2002), and the swelling response to subcutaneous injection of PHA (expressed in mm $\times 10^2$) was assumed to represent a reliable indicator of the T-cell mediated immunity (Martin et al. 2006; for further details, see Romano et al. 2008). Although the interpretation of the "PHA test" has been questioned, its reliability to evaluate T-cell mediated immunocompetence has been validated recently (see Tella et al. 2008). Blood samples were collected in capillary tubes by puncturing the ulnar vein as soon as the chick was found hatched to perform molecular sexing (Rubolini, Romano, Martinelli, and Saino 2006). The plasma was separated after centrifugation for 10 min and stored at -20 °C for AOC and ROM analyses (see below).

In the present study, we included only broods where at least 2 chicks survived until the age of 9 days. This selection of broods resulted in the inclusion of 186 chicks in the analyses of redox status at age 9 days. Sample sizes for the analyses at age 1 are slightly smaller (see RESULTS) mainly because of accidental loss of blood samples. For all these chicks, the measure of tarsus length and body mass was available. The measure of the swelling response to PHA was available for 177 chicks because some chicks could not be found, had been preyed, or had died for other reasons on the day between PHA injection and response measurement.

Plasma AOC and ROM concentration were measured on blood samples collected around hatching, when blood for molecular sexing was also collected (see above), and at age 9 days. Because blood at age 9 was sampled before starting the immune response test, the analyses address the question of whether redox status influenced immunity rather than whether immune response affected redox status (see Costantini and Møller 2009).

Molecular sexing was performed according to the protocol originally devised by Griffiths et al. (1996), as modified according to Saino, Martinelli, and Romano (2008). Reliability of the method was confirmed previously (Rubolini, Romano, Martinelli, and Saino 2006).

Plasma AOC and ROM

The plasma antioxidant barrier includes both exogenous (e.g., ascorbate, tocopherols, carotenoids) and endogenous (e.g., uric acid, enzymes) compounds. Because of synergistic and antagonistic effects of different antioxidants, total AOC afforded by the multifaceted antioxidant system of vertebrates is not a simple additive function of the concentration of individual antioxidants, and overall measures of AOC are thus more representative of the redox status of an individual (Cohen et al. 2007; Monaghan et al. 2009). For this reason, the effect of lutein treatment was assessed on an overall index of plasma AOC. The total AOC of plasma was measured using the OXY-Adsorbent test (Diacron, Grosseto, Italy) (for application of the same protocol, see Costantini and Dell'Omo 2006; Costantini, Cardinale, and Carere 2007; Costantini, Coluzza, et al. 2007; Costantini, Fanfani, and Dell'Omo 2007). This test uses a colorimetric determination to quantify the ability of the plasma antioxidant barrier to cope with the oxidant action of hypochlorous acid (HClO). The plasma (5 µl) was diluted 1:100 with distilled water. A 5 µl aliquot of

the diluted plasma was added to 200 µl of a titered HClO solution. The solution was gently mixed and incubated for 10 min at 37 °C. At the end of the incubation time, 5 µl of an alkyl-substituted aromatic amine solubilized in a chromogenic mixture was added. Such amine is oxidized by the residual HClO and transformed in a pink-colored derivative. The concentration of colored complex is directly proportional to the HClO excess and inversely related to the AOC of tested plasma. The intensity of the colored solution was measured at 492 nm using a photometer (Multiskan EX; Labsystem, Franklin, MA). One standard sample of known AOC and one blank sample (5 µl of distilled water) were processed and used as reference. Repeatability of AOC measures was tested on 21 (age 1) or 42 (age 9) samples that were assayed in duplicate and was found to be large and highly significant (repeatability according to Lessells and Boag (1987): age 1: 0.580, z = 2.25, $P = 0.012$; age 9: 0.560, z = 3.14, $P < 0.001$).

ROM are markers of early oxidative damage. ROM (mainly hydroperoxides, ROOH) represent the primary products of the oxidative cascade derived from the oxidation of biomolecules, particularly lipids (e.g., Porter et al. 1995), caused by exposure to ROS. ROM are more stable than ROS and therefore they can be detected and quantified and have been used as a marker of early oxidative damage (i.e., damage of biomolecules early in the oxidation cascade) in several recent studies (see Costantini and Dell'Omo 2006; Costantini, Cardinale, and Carere 2007; Costantini, Coluzza, et al. 2007; Costantini, Fanfani, and Dell'Omo 2007; Bonisoli-Alquati et al. 2010; Costantini and Bonadonna 2010; see also Monaghan et al. 2009). Besides being the product of oxidation of biomolecules, ROM are themselves pro-oxidants and can therefore further propagate the oxidation chain reaction (Halliwell and Gutteridge 2007). The plasma concentration of ROM was measured by the d-ROM test (Diacron; Grosseto, Italy; see also Costantini, Cardinale, and Carere 2007 and references therein). The plasma (10 µl) was diluted with 200 µl of a solution containing an acetate buffer (pH 4.8) and an alkyl-substituted aromatic amine solubilized in a chromogenic mixture. The solution was gently mixed and then incubated for 75 min at 37 °C. During incubation, the acidic pH of the acetate buffer favored the iron release from plasma proteins. This metal catalyzed the cleavage of ROOH in 2 different free radicals. Such radicals are able to oxidize the alkyl-substituted aromatic amine solubilized in the chromogen producing a pink-colored derivative whose color intensity is directly proportional to the concentration of ROM. After incubation, the absorbance was read at 492 nm using a photometer (Multiskan EX; Labsystem). One standard sample and one blank sample (10 µl of distilled water) were processed and used as reference. Repeatability of ROM measures was tested on 21 (age 1) or 22 (age 9) samples that were assayed in duplicate and was also found to be large and highly significant (repeatability: age 1: 0.826, $z = 2.85$, $P = 0.002$; age 9: 0.762, $z = 2.78$, $P = 0.003$).

In all analyses, statistics for AOC are given as millimoles per liter of HClO neutralized and for ROM as millimoles per liter of H_2O_2 equivalents.

Statistical analyses

We relied on linear mixed-effects models (LMMs) to analyze chick phenotypic traits. In all analyses, degrees of freedom were estimated using the Kenward–Rogers method as implemented in PROC MIXED of SAS 9.1 (Littell 1996), and nest was included as a random factor. Egg treatment, sex of the chick, and laying order were considered as fixed effect factors, and laying date of the original egg and egg mass were included as covariates in the analyses where these effects were investigated. The main focus of the study was on the effect of lutein treatment on physiological variables (AOC and ROM) as well as on the covariation of body size and immune response with these variables in relation to egg treatment, sex, laying order, and their combined (interaction) effects. The main analyses presented in the RESULTS therefore consist of LMM with nest as random factor and up to 3-way interactions among treatment, sex, laying order, as well as AOC or ROM (in the analyses of covariation with tarsus length or immune response). Age (in days from hatching) was included as a covariate to account for small variation in actual age with respect to age when measurement had been planned (see above). This design led to complex models including 16 terms. To simplify these models, we first removed all 3-way interactions at once whenever none of them was significant. When significant 2-way interactions emerged, the simplification procedure was stopped at this stage and the significance of the 2-way interaction terms was tested in these reduced models. This procedure allowed us to reduce model complexity without largely inflating type I error rate due to multiple statistical tests (Wittingham et al. 2006). However, following Nakagawa (2004), we did not apply any correction of the α value in multiple tests. When there were no significant 2-way interactions, all interaction terms were also removed from the model. To describe the differential pattern of variation of tarsus length or immune response according to AOC or ROM, however, linear models were applied which included the significant interactions and the relevant main effects, along with the main effects not involved in the significant interactions. This allowed us to obtain parameter estimates for the relationship between tarsus length or immune response and AOC or ROM in all cases as these covariates were involved in only one significant 2- or 3-way interaction in all cases.

To reduce the complexity of the results, we did not duplicate the analyses of tarsus length on body mass data. This was decided because tarsus length and body mass are strongly positively correlated and, in the present context, we assumed that both mainly reflect overall body size. In fact, a linear model of body mass with treatment, sex, laying order, and age as covariates showed a highly significant positive effect of tarsus length on body mass ($F_{1,97} = 230.99$, $P < 0.001$) and that tarsus length explained a large proportion of the variance in body mass (partial $\eta^2 = 0.704$). Moreover, a model with up to 3-way interactions among covariates showed that the pattern of covariation between body mass and tarsus length did not depend on treatment, sex or laying order. We preferred to use tarsus length rather than body mass because its measure is not affected by food provisioning around the time of measurement, which can introduce considerable noise in body mass data owing to the large size of prey items (e.g., relatively large fish) provided on occasions by parents.

Estimates of statistical parameters are given with their standard error in parentheses.

RESULTS

Plasma AOC and ROM in relation to treatment, sex and laying order

The effects of lutein injection in the yolk on plasma AOC and ROM at hatching were assayed in 182 and 183 chicks, respectively, from 82 broods (see Figure 1 for mean values of the treatment, sex and laying order groups, and sample sizes; see MATERIALS AND METHODS for justification of the small difference in sample sizes between age 1 and 9). Linear mixed models showed no significant effects of 3- or 2-way interactions of treatment, sex or laying order on either variable ($P > 0.05$ in all cases, details not shown). The main effects of all factors were also nonsignificant (Table 1). Similarly, AOC at age 9, which was measured in 186 chicks from 83 broods, was not predicted by the interaction or main effects of treatment, sex or laying order

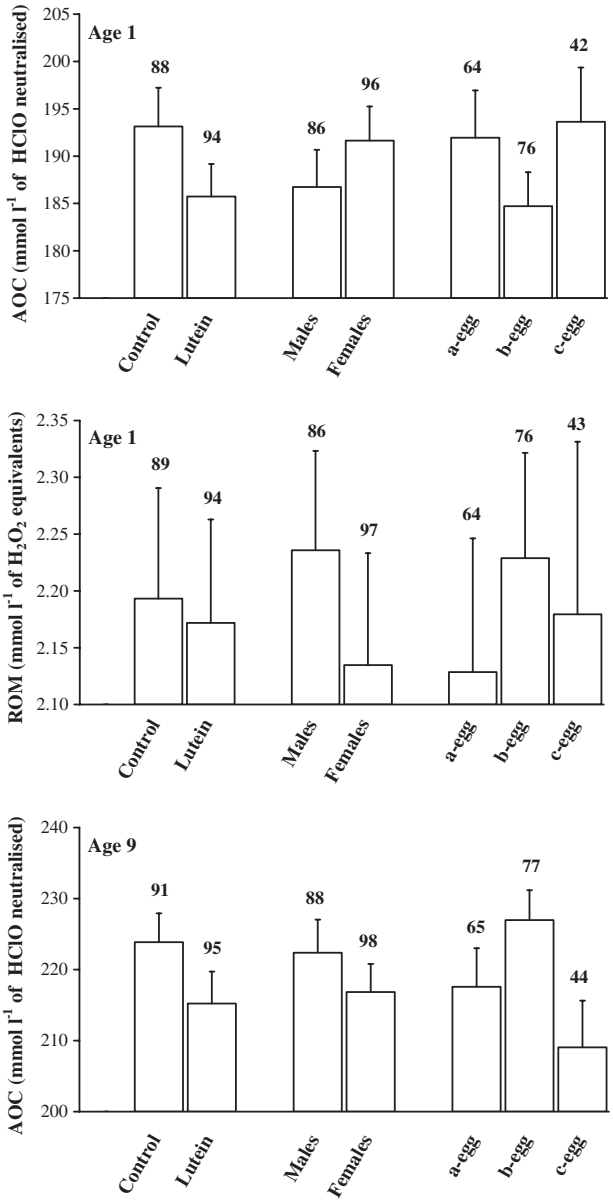

Figure 1
Mean (+standard error) plasma AOC at age 1 and 9 days after hatching, and plasma ROM concentration at age 1 day in the treatment, sex or laying order groups. Sample size for individual groups is reported.

(Table 1; Figure 1). The models including laying date or egg mass also failed to disclose any significant interaction or main effect after model simplification (see Statistical analyses).

In the analyses of ROM at age 9, however, the 2-way interaction terms between egg treatment and sex or laying order significantly predicted ROM (Table 1). Among chicks from lutein eggs, males had larger ROM than females, whereas no sex-related variation existed among control chicks (Figure 2). Moreover, the concentration of ROM was larger among lutein than control males, albeit the difference was marginally non-significant (post hoc test, $P = 0.069$), whereas the effect of treatment was far from statistically significant among females (Figure 2).

ROM did not vary with laying order among control chicks as the larger concentration recorded in c-eggs did not differ statistically from that in either a- or b-eggs (Figure 2). Among

Table 1
Linear mixed model of AOC or plasma ROM concentration at age 1 or 9 days after hatching in relation to treatment of the original egg, sex and laying order

	AOC			ROM		
	F/*z*	df	*P*	*F*/*z*	df	*P*
Age 1						
Brood	/			0.86		0.194
Treatment	2.71	1, 177	0.102	0.01	1, 129	0.915
Sex	1.22	1, 177	0.272	0.48	1, 169	0.494
Laying order	1.35	2, 177	0.261	0.17	2, 127	0.842
Age 9						
Brood	/			1.65		0.050
Treatment	1.25	1, 176	0.266	0.09	1, 149	0.765
Sex	1.03	1, 176	0.311	4.43	1, 158	0.039
Laying order	2.63	2, 176	0.075	1.52	2, 124	0.227
Treatment × sex	0.26	1, 176	0.613	5.50	1, 166	0.022
Treatment × laying order	0.07	2, 176	0.933	5.76	2, 158	0.004
Sex × laying order	0.31	2, 176	0.736	2.34	2, 172	0.104

F values are reported for fixed effects and *z* values for the random effect of brood. Exclusion of the 2-way interactions from the model of AOC at age 9 did not disclose any significant main effect. df, degrees of freedom.

lutein chicks, those originating from c-eggs had smaller ROM values than those from the previous eggs. As a result of the differential covariation of ROM with laying order, mean ROM of lutein a-chicks was larger and that of lutein c-chicks was

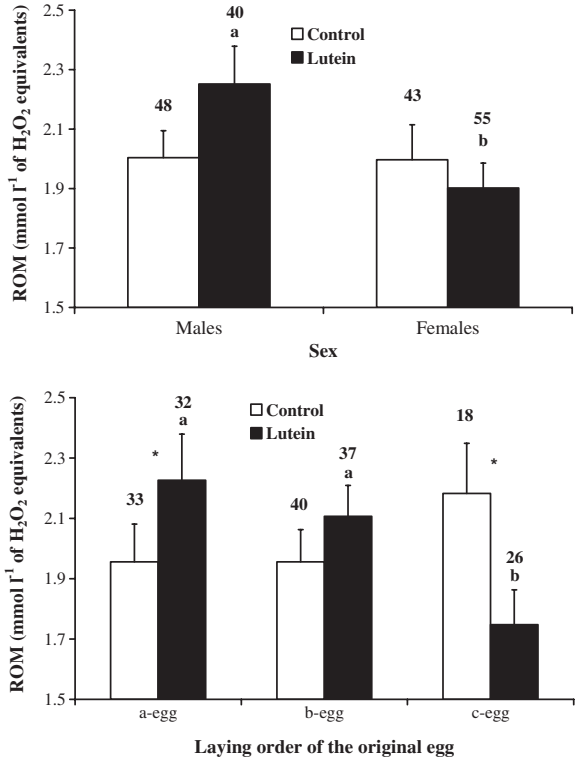

Figure 2
Mean (+standard error) ROM concentration at age 9 days in relation to egg lutein treatment and sex (upper panel) or lutein treatment and laying order (lower panel). Different letters indicate a significant ($P < 0.05$ at post hoc tests) difference between laying order positions within treatment groups. Asterisks indicate significant difference between treatment groups within laying order positions. Sample size for individual groups is reported.

smaller than mean ROM recorded for control chicks of the corresponding laying order (Figure 2). As observed for AOC, ROM was not significantly predicted by egg mass and the interactions with treatment, sex and laying order. There was a marginally significant interaction between treatment, laying order, and laying date ($F_{2,150} = 3.16$, $P = 0.045$). The relationship between ROM and date was positive for a- and c- and negative for b-chicks from the control group, whereas the opposite pattern of variation in the slope of this relationship was observed among lutein chicks. However, as none of the coefficients estimated for the treatment by laying order groups significantly deviated from 0 and the effect of date was not at the focus of the study, this complex interaction effect will not be discussed further.

Covariation between plasma AOC and ROM

The covariation of ROM with AOC at either age was analyzed in models including up to 3-way interactions of treatment, sex and laying order as well as AOC.

At age 1, a significant 3-way interaction effect on ROM emerged between sex, laying order, and AOC ($F_{2,158} = 3.41$, $P = 0.036$). ROM increased with AOC among male a-chicks (coefficient = 0.016 (0.006), $t_{155} = 2.76$, $P = 0.007$), whereas it did not covary with AOC in the other sex by laying order groups ($P > 0.115$). The other 3-way interactions did not predict ROM (details not shown).

At age 9, ROM was not predicted by 3- or 2-way interactions between treatment, sex, or laying order on the one side, and AOC. After exclusion of the nonsignificant 3- and 2-way interactions, the simplified model demonstrated a significant positive covariation between ROM and AOC (coefficient for AOC = 0.0029 (0.0012), $F_{1,170} = 5.63$, $P = 0.019$; Figure 3; other details not shown) and confirmed the same significant interactions as in Table 1.

Overall, AOC values were significantly larger at age 9 than around hatching (paired *t*-test; mean AOC at age 1: 189.3 (2.66); age 9: 219.4 (3.09); $t_{181} = 7.06$, $P < 0.001$). However, the increase in AOC did not depend on egg treatment, sex or laying order, as shown by the nonsignificant effect of interactions of these factors with AOC at age 1 in linear mixed models.

ROM at age 1 was marginally nonsignificantly larger than at age 9 (age 1: 2.18 (0.066); age 9: 2.03 (0.052); $t_{182} = 1.89$, $P = 0.060$). In a mixed model of ROM at age 9 from which 3-way interactions among ROM at age 1, treatment, sex and laying order were excluded as nonsignificant, a significant effect of the 2-way interaction between sex and ROM at age 1 emerged ($F_{1,167} = 7.37$, $P = 0.007$), with ROM at age 9 increasing with ROM at age 1 among females (coefficient = 0.113 (0.127)) and decreasing among males (−0.208 (0.139)). In this model, the 2-way interactions between treatment and sex or laying order that were found to significantly predict ROM at age 9 (Table 1) were still significant ($P < 0.031$; details not shown).

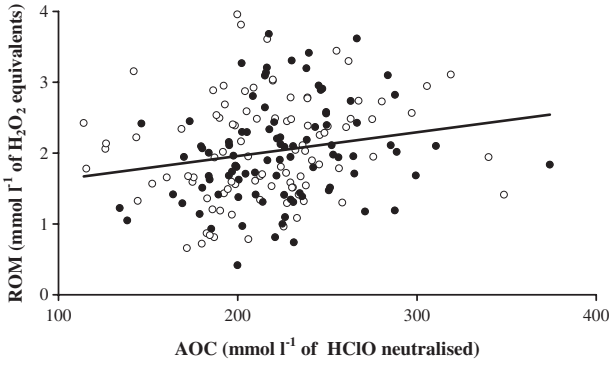

Figure 3
Covariation between plasma ROM and AOC of individual chicks at age 9 days. Full circles: controls; open circles: lutein treated. Regression line is fitted to all data points, irrespective of treatment.

AOC and lutein treatment as predictors of chick phenotype

At hatching, chick tarsus length was positively predicted by plasma AOC in a model including the main effects of treatment, sex, laying order as well as age at measurement (Table 2; Figure 4). Male chicks were significantly larger than females (estimated marginal means (EMM) of males: 27.5 (0.13), females: 27.2 (0.13), and chicks from a-eggs or b-eggs (EMM: 27.8 (0.14) and 27.7 (0.13), respectively) were significantly larger than those from c-eggs (26.7 (0.17); post hoc tests, $P < 0.001$ in both comparisons). The 2- and 3-way interactions were removed from the model as they were nonsignificant (details not shown). Inclusion of egg size, which is a strong predictor of body size at hatching, did not qualitatively alter the results (effect of AOC at day 1: $F_{1,158} = 4.73$, $P = 0.031$, coefficient = 0.005 (0.002)).

In a factorial model, 3-way interactions among treatment, sex, laying order, and AOC did not significantly predict tarsus length at age 9. In a simplified model including up to second-order interactions, the significant effect of the treatment by AOC interaction indicated that the covariation between body size and plasma AOC depended on the original egg being injected or not with lutein (Table 3). The slopes of the relationships between size and AOC in either treatment group were investigated in a model including the AOC, treatment, and their interaction, together with the main effects of sex, laying order, and age. This model showed no significant variation of tarsus length with AOC among controls ($t_{165} = 1.12$, $P = 0.265$, coefficient = 0.010 (0.009)), whereas a marginally nonsignificant decline with AOC emerged for lutein chicks ($t_{152} = -1.83$, $P = 0.070$, coefficient = −0.0137 (0.0075)).

The covariation between immune response and AOC at age 9 depended on the combined effects of sex and laying order (Table 3). A model including the main and up to third-order interaction effects of AOC, sex and laying order, together with age showed that immune response increased with AOC among male a-chicks (coefficient = 0.449 (0.136); $t_{156} = 3.32$, $P = 0.001$) and among female b-chicks (coefficient = 0.375 (0.144); $t_{155} = 2.60$, $P = 0.010$). The slopes of the relationships within the other sex by laying order groups were nonsignificant ($P > 0.200$). Immune response increased with AOC more among male than female a-chicks (coefficient for female a-chicks = 0.0009 (0.146), difference: $t_{156} = 2.25$, $P = 0.026$), whereas the reverse was true among b-chicks (cofficient for male b-chicks: −0.194 (0.161), difference: $t_{155} = 2.63$, $P = 0.009$).

Table 2
Linear mixed model of tarsus length at hatching in relation to treatment, sex, laying order, and AOC, where exact age at measurement was included as a covariate

	F/*z*	df	*P*
Brood	3.47		<0.001
Treatment	2.41	1, 107	0.123
Sex	4.04	1, 137	0.046
Laying order	16.41	2, 111	<0.001
AOC[a]	7.17	1, 149	0.008
Age	51.88	1, 162	<0.001

Interaction terms were removed as they were nonsignificant.
[a] The coefficient for AOC was 0.006 (0.002).

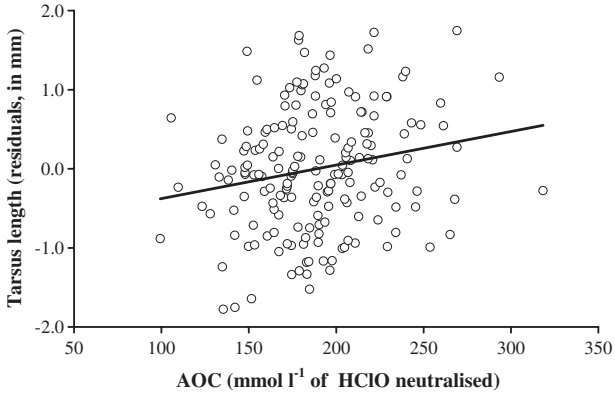

Figure 4
Residuals of tarsus length measured at age 1 in relation to plasma AOC at the same age. Residuals are obtained from a model including the main effects of treatment, sex, laying order, and age at measurement.

ROM and lutein treatment as predictors of chick phenotype

At hatching, ROM did not predict tarsus length either in combination with other factors or in a simplified model including only the main effects of treatment, sex, laying order, and age (effect of ROM in the simplified model: $F_{1,156} = 0.01$, $P = 0.914$).

Tarsus length measured at the age of 9 days was predicted by ROM but differentially so according to egg treatment and laying order (Table 4). Tests on the slopes of the relationships between tarsus length and ROM in the 6 sex by laying order groups were based on a model that included the relevant terms, as well as the main effects of sex and age. Tarsus length significantly increased with ROM among control chicks from b-eggs ($t_{146} = 2.86$; $P = 0.005$, coefficient = 2.047 (0.716)) or c-eggs ($t_{143} = 3.21$, $P = 0.002$, coefficient = 3.329 (1.039)) but not a-eggs ($t_{143} = -1.25$, $P = 0.212$), whereas the slope of the relationship between tarsus length and ROM did not differ from 0 among lutein chicks of all laying orders ($P > 0.600$; Figure 5). The slope of these relationships did not differ between lutein and control chicks originating from either a- or b-eggs ($P > 0.130$ in both cases). However, tarsus length increased more with ROM among control than lutein c-chicks ($t_{143} = 2.01$, $P = 0.046$).

Similarly to tarsus length, immune response was also significantly affected by ROM in a differential way according to laying order and treatment (Table 3). In a model with the main effects of treatment, sex, laying order, and ROM and up to 3-way interactions between treatment, laying order, and ROM, immune response of lutein a-chicks was positively predicted by ROM (coefficient = 21.07 (7.591), $t_{158} = 2.78$, $P = 0.006$), whereas no significant covariation with ROM existed for the other laying order by treatment groups ($P > 0.088$ in all cases). However, a significant difference in the slope of the relationship between immune response and ROM existed for b-chicks as the slope for controls was larger than that for lutein chicks (controls: coefficient = 14.90 (8.697); lutein chicks: coefficient = −15.49 (9.881); $t_{156} = 2.31$, $P = 0.022$).

Table 3
Linear mixed models of tarsus length or immune response at age 9 in relation to egg treatment, sex, laying order, and plasma AOC

	Tarsus length			Immune response		
	F/*z*	df	*P*	*F*/*z*	df	*P*
Brood	3.72		<0.001	2.24		0.013
Treatment	5.06	1, 151	0.026	0.00	1, 148	0.989
Sex	0.48	1, 145	0.491	0.07	1, 134	0.785
Laying order	2.53	2, 146	0.083	0.31	2, 146	0.736
AOC	0.05	1, 145	0.831	3.43	1, 149	0.066
Treatment × sex	0.47	1, 140	0.496	0.26	1, 142	0.618
Treatment × laying order	2.42	2, 156	0.092	0.90	2, 144	0.410
Sex × laying order	2.18	2, 149	0.116	4.90	2, 147	0.009
AOC × treatment	5.23	1, 152	**0.024**	0.00	1, 148	0.998
AOC × sex	0.02	1, 146	0.891	0.17	1, 136	0.682
AOC × laying order	0.96	2, 146	0.385	0.41	2, 147	0.667
Treatment × sex × lying order				0.56	2, 145	0.573
AOC × treatment × sex				0.03	1, 143	0.864
AOC × treatment × laying order				1.31	1, 145	0.274
AOC × sex × laying order				5.80	2, 146	**0.004**
Age	17.88	1, 157	<0.001	0.42	1, 153	0.518

F values are reported for fixed effects and *z* values for the random effect of brood. See MATERIALS AND METHODS for simplification procedures of the models. Significant tests ($P < 0.05$) are in bold.

DISCUSSION

We tested whether a physiological increase in the concentration of lutein in yellow-legged gull eggs affected plasma AOC and oxidative damage (ROM) in early life stages. This hypothesis was based on the physiological actions of carotenoids (Surai 2002), on the observation that yolk carotenoids prevail over dietary ones for long after hatching (Karadas et al. 2005), and on suboptimal egg carotenoid content due to limitation in maternal diet (Blount, Surai, Nager, et al. 2002). The decline in antioxidants with laying order (Saino, Bertacche, et al. 2008; Karadas F, Rubolini D, unpublished data) and sex-biased susceptibility to carotenoids (Romano

Table 4
Linear mixed models of tarsus length or immune response in relation to egg treatment, sex, laying order, and plasma ROM

	Tarsus length			Immune response		
	F/*z*	df	*P*	*F*/*z*	df	*P*
Brood	3.37		<0.001	1.47		0.071
Treatment	4.04	1, 131	0.046	0.00	1, 142	0.999
Sex	0.49	1, 143	0.486	1.16	1, 153	0.283
Laying order	9.05	2, 129	<0.001	0.67	2, 144	0.515
ROM	3.52	1, 146	0.063	0.72	1, 151	0.398
Treatment × sex	1.55	1, 127	0.216	2.21	1, 146	0.139
Treatment × laying order	1.82	2, 145	0.166	2.53	2, 153	0.083
Sex × laying order	1.18	2, 135	0.311	0.23	2, 147	0.794
ROM × treatment	4.38	1, 130	0.038	0.00	1, 141	0.966
ROM × sex	0.03	1, 141	0.856	1.23	1, 152	0.269
ROM × laying order	2.65	2, 131	0.074	0.80	2, 146	0.451
Treatment × sex × lying order	0.49	2, 137	0.612	0.26	2, 147	0.769
ROM × treatment × sex	1.19	1, 130	0.277	1.50	1, 148	0.222
ROM × treatment × laying order	3.38	2, 144	**0.037**	3.81	2, 154	**0.024**
ROM × sex × laying order	1.05	2, 137	0.352	0.83	2, 149	0.438
Age	22.32	1, 153	<0.001	0.47	1, 151	0.495

F values are reported for fixed effects and *z* values for the random effect of brood.

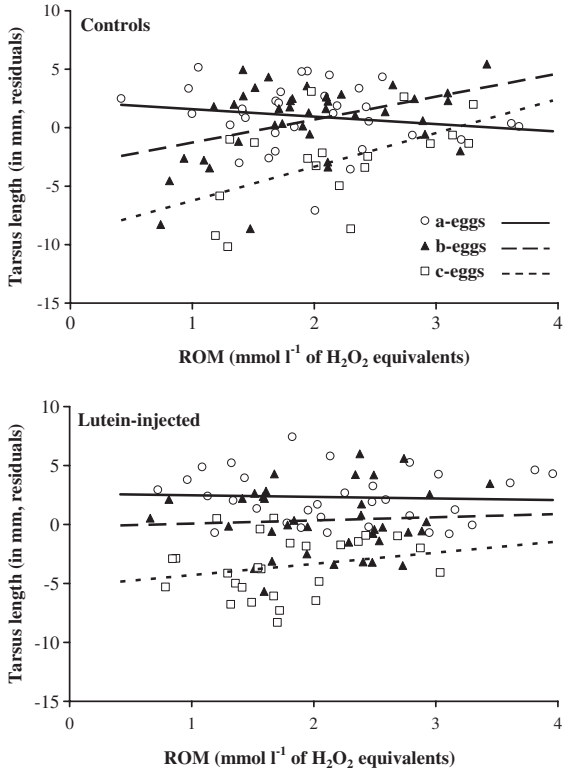

Figure 5
Tarsus length at age 9 days in relation to plasma ROM concentration at the same age in control chicks or chicks from lutein-injected eggs according to laying order.

et al. 2008) led us to test whether lutein differently affected the redox status of chicks of different sex or laying order.

The experimental design we adopted differs from previous studies of the effect of egg carotenoids on redox status in that we administered carotenoids directly into the egg, thus avoiding any effect of dietary carotenoids on aspects of egg quality other than carotenoid content, and mediated by maternal physiology.

Effects of lutein on redox status and covariation between ROM and AOC

Lutein did not affect plasma AOC. This finding corroborates the claim that carotenoids do not substantially contribute to antioxidant defense in birds (Hartley and Kennedy 2004; Costantini and Møller 2008) but does not preclude that antioxidant defense was enhanced in target tissues other than blood. No effect of lutein emerged on ROM at hatching. At age 9 days, however, ROM did not differ between male and female controls but was significantly larger in lutein males compared with lutein females and control males. These results also contradict the idea that lutein provides protection against oxidation, as this should have resulted in reduced ROM among lutein chicks. Moreover, they provide further evidence for sex-biased sensitivity to maternal effects of carotenoids (De Neve et al. 2008; Romano et al. 2008) and for effects on chick physiology enduring well after hatching (Koutsos et al. 2003).

Carotenoids may act as pro-oxidants (Palozza 1998; El-Agamey et al. 2004) and increased carotenoid concentration may thus have had pro-oxidant effects in males, although this interpretation is contradicted by the fact that carotenoid concentrations within the natural range of variation are far from those having pro-oxidant effects (Surai 2002).

Carotenoids may not be major contributors to AOC but may be an important component of an integrated system where individual components interact synergistically or by mutual recycling with other antioxidants (Surai 2002). An imbalance among the compartments of this integrated system could have detrimental consequences. The relative concentrations of carotenoids and other antioxidants in the yellow-legged gull, however, vary widely among eggs (Saino, Bertacche, et al. 2008; Karadas F, Rubolini D, unpublished data), and the carotenoid solution we injected also contained minor amounts of zeaxanthin, which is the second most abundant carotenoid in yellow-legged gull eggs, suggesting that the minor change in the relative carotenoid concentrations we applied should not have caused any serious imbalance. An alternative interpretation hints at the complex functions accomplished by carotenoids, other than antioxidant protection. Carotenoids have a multitude of roles in cell–cell communication, regulation of gene expression, cell differentiation, and on the immune system (Møller et al. 2000; Surai 2002). Lutein may have promoted any of these functions causing increased metabolism and thus higher levels of ROM. For example, an upregulation of the immune system may increase ROS production because phagocytes and lymphocytes release ROS. Males may be more sensitive to these effects because they are more susceptible to parasitism already during early life stages (Tschirren et al. 2003). This scenario is compatible with the tenet that dietary carotenoids are limiting (Olson and Owens 1998) and suggests that yolk carotenoids enhance specific physiological processes, at the cost of increased oxidative damage.

While at age 9 days, AOC showed a decline, though not significant, with laying order independently of egg treatment, in line with previous findings (Rubolini, Romano, Bonisoli-Alquati, and Saino 2006), oxidative damage was significantly increased by lutein treatment in a-chicks and the reverse was true for c-chicks. In the same population, carotenoid concentration is smaller in c- than in a-eggs (Saino, Bertacche, et al. 2008; Karadas F, Rubolini D, unpublished data). Lutein may have promoted antioxidant defense of carotenoid-depleted c-eggs, causing lower ROM compared with control c-chicks. However, larger ROM in lutein than in control a-chicks cannot be reconciled with antioxidant lutein effects, and alternative interpretations as outlined for the effects of lutein on ROM in males may be invoked, if carotenoids are limiting to c- but not a-egg quality.

The analyses of AOC controlling for ROM confirmed the absence of effects of treatment. Reciprocally, the analyses of ROM controlling for AOC confirmed the significant treatment by sex or laying order effects, implying that differential effects of lutein according to sex or laying order existed independently of plasma AOC.

If lutein as an antioxidant interferes with the balance between oxidative damage and antioxidant defense, a given level of ROM should be associated with higher AOC in lutein than in control chicks. Contrasting with this expectation, the effect of the interaction between egg treatment and AOC on ROM was nonsignificant, again arguing against a major antioxidant action of lutein. Independently of the effect of treatment, depletion of circulating antioxidants should be accompanied by low ROM. A positive covariation between AOC and ROM indeed emerged, at age 9 days only, thus suggesting that depletion of antioxidants occurred some days after hatching.

Effects of lutein on covariation between size, immune response, and redox status

The second aim of the study was to test whether lutein supplementation interfered with the resolution of trade-offs between growth or immune response and AOC or ROM. Body size

at hatching was positively predicted by AOC, consistently with the prediction that high AOC has beneficial effects on embryonic growth (see Rubolini, Romano, Bonisoli-Alquati, and Saino 2006). At age 9 days, however, the significant covariation between body size and AOC did not persist among controls. Among lutein chicks, on the other hand, there was a nonsignificant decline of tarsus length with increasing AOC, resulting in a significant treatment by AOC interaction. The covariation of tarsus length at age 9 and AOC among lutein chicks was paralleled by no significant covariation with ROM. On the other hand, among control chicks from b- and c-eggs, tarsus length increased with ROM. Hence, lutein interfered with the resolution of a possible trade-off between redox status and growth. Among lutein chicks, larger growth was attained to the expense of AOC, with no cost in terms of oxidative damage, whereas among b- and c-control chicks, larger body size had no detrimental consequences for AOC but resulted in larger ROM. The nonsignificant covariation between tarsus length and ROM among control a-chicks could result from larger lutein concentration in a-eggs, and thus on negligible effects of supplemental lutein.

We also tested whether the covariation between immune response to PHA and AOC or ROM depended on egg lutein. The covariation between immune response and ROM depended on treatment and laying order. The relationship between immune response and ROM was positive for lutein a-chicks, but among b-chicks, the coefficient was negative and smaller for lutein than for control chicks. This suggests that lutein had immunomodulating effects which depended on chick oxidative status but also on quality and nutrition of the chick as affected by laying and hatching order.

In conclusion, we analyzed the consequences of variation in egg carotenoid concentration in the eggs for antioxidant defense and oxidative damage of the offspring of either sex by direct manipulation of yolk carotenoids. Lutein raised oxidative damage of male chicks and of first laid offspring. As the lutein dose was within physiological limits, these results suggest that lutein upregulated physiological functions resulting in higher oxidative damage. Moreover, lutein interfered with the resolution of trade-offs between growth or immunity and redox status. Hence, variation in yolk carotenoid concentration may have differential consequences on redox physiological status of male and female offspring. Overall, we found no evidence that yolk lutein enhanced chick antioxidant plasma levels, corroborating claims that carotenoids are minor components of birds antioxidant system. Still, present results show that carotenoids retain important effects on individual oxidative stress, whose mechanisms still need to be uncovered.

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REFERENCES

Alonso-Alvarez C, Bertrand S, Devevey G, Gaillard M, Prost J, Faivre B, Sorci G. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat. 164:651–659.

Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. Proc R Soc Lond B Biol Sci. 274:819–825.

Badyaev AV. 2002. Growing apart: an ontogenetic perspective on the evolution of sexual size dimorphism. Trends Ecol Evol. 17:369–378.

Badyaev AV. 2008. Maternal effects as generators of evolutionary change: a reassessment. In: Schlichting CD, Mousseau TA, editors. The year in evolutionary biology 2008. New York: Wiley-Blackwell. p. 151–161.

Badyaev AV, Acevedo Seaman D, Navara KJ, Hill GE, Mendoca MT. 2006. Evolution of sex-biased maternal effects in birds: III. Adjustment of ovulation order can enable sex-specific allocation of hormones, carotenoids, and vitamins. J Evol Biol. 19:1044–1057.

Badyaev AV, Schwabl H, Young RL, Duckworth RA, Navara K, Parlow AF. 2005. Adaptive sex differences in growth of pre-ovulation oocytes in a passerine bird. Proc R Soc Lond B Biol Sci. 272:2165–2172.

Badyaev AV, Uller T. 2009. Parental effects in ecology and evolution: mechanism, processes and implications. Philos Trans R Soc B Biol Sci. 364:1169–1177.

Balaban RS, Nemoto S, Finkel T. 2005. Mitochondria, oxidants, and aging. Cell. 120:483–495.

Beckman KB, Ames BN. 1998. The free radical theory of aging matures. Physiol Rev. 78:547–581.

Bertrand S, Alonso-Alvarez C, Devevey G, Faivre B, Prost J, Sorci G. 2006. Carotenoids modulate tradeoffs between egg production and resistance to oxydative stress in zebra finch. Oecologia. 147: 576–584.

Biard C, Gil D, Karadas F, Saino N, Spottiswoode CN, Surai PF, Møller AP. 2009. Maternal effects mediated by antioxidants and the evolution of carotenoid-based signals in birds. Am Nat. 174:696–708.

Biard C, Surai PF, Møller AP. 2007. An analysis of pre- and post-hatching maternal effects mediated by carotenoids in the blue tit. J Evol Biol. 20:326–339.

Blount JD, Houston DC, Surai PF, Møller AP. 2004. Egg-laying capacity is limited by carotenoid pigment availability in wild gulls *Larus fuscus*. Proc R Soc Lond B Biol Sci. 271:S79–S81.

Blount JD, Surai PF, Houston DC, Møller AP. 2002. Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. Funct Ecol. 16:445–453.

Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, Trewby M, Kennedy MW. 2002. Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proc R Soc Lond B Biol Sci. 269:29–36.

Bonisoli-Alquati A, Mousseau TA, Møller AP, Caprioli M, Saino N. 2010. Increased oxidative stress in barn swallows from the Chernobyl region. Comp Biochem Physiol A. 155:205–210.

Chew BP, Park JS. 2004. Carotenoid action on the immune response. J Nutr. 134:257S–261S.

Cohen A, Klasing K, Ricklefs R. 2007. Measuring circulating antioxidants in wild birds. Comp Biochem Physiol B. 147:110–121.

Costantini D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett. 11:1238–1251.

Costantini D, Bonadonna F. 2010. Patterns of variation of serum oxidative stress markers in two seabird species. Polar Res. 29:30–35.

Costantini D, Cardinale M, Carere C. 2007. Oxidative damage and anti-oxidant capacity in two migratory bird species at a stop-over site. Comp Biochem Physiol C. 144:363–371.

Costantini D, Coluzza C, Fanfani A, Dell'Omo G. 2007. Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). J Comp Physiol B. 177:723–731.

Costantini D, Dell'Omo G. 2006. Effects of T-cell mediated immune response on avian oxidative stress. Comp Biochem Physiol A. 145: 137–142.

Costantini D, Fanfani A, Dell'Omo G. 2007. Carotenoid availability does not limit the capability of nestling kestrels (*Falco tinnunculus*) to cope with oxidative stress. J Exp Biol. 210:1238–1244.

Costantini D, Møller AP. 2008. Carotenoids are minor antioxidants for birds. Funct Ecol. 22:367–370.

Costantini D, Møller AP. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. Comp Biochem Physiol A. 153: 339–344.

Cramp S. 1998. The complete birds of the Western Palearctic on CD-ROM. Oxford: Oxford University Press.

De Neve L, Fargallo JA, Vergara P, Lemus JA, Jarén-Galán M, Luaces I. 2008. Effects of maternal carotenoid availability in relation to sex,

parasite infection and health status of nestling kestrels (*Falco tinnunculus*). J Exp Zool. 211:1414–1425.
Edge R, McGarvey DJ, Truscott TG. 1997. The carotenoids as antioxidants—a review. J Photochem Photobiol B. 41:189–200.
Eising CM, Eikenaar C, Schwabl H, Groothuis TGG. 2001. Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. Proc R Soc Lond B Biol Sci. 268:839–846.
El-Agamey A, Lowe GM, McGarvey DJ, Mortensen A, Philip DM, Truscott TG, Young AJ. 2004. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. Arch Biochem Biophys. 430:37–48.
Ewen JG, Thorogood R, Brekke P, Cassey P, Karadas F, Armstrong DP. 2009. Meternally invested carotenoids compensate costly ectoparasitism in the hihi. Proc Natl Acad Sci U S A. 106:12798–12802.
Ewen JG, Thorogood R, Karadas F, Pappas AC, Surai PF. 2006. Influences of carotenoid supplementation on the integrated antioxidant system of a free living endangered passerine, the hihi (*Notiomystis cincta*). Comp Biochem Physiol A. 143:149–154.
Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. Nature. 408:239–247.
Fitze PS, Tschirren B, Richner H. 2003. Carotenoid-based colour expression is determined early in nestling life. Oecologia. 137:148–152.
Goodwin TW. 1984. The biochemistry of the carotenoids. Volume II. Animal. London: Chapman & Hall.
Griffiths R, Daan S, Dijkstra C. 1996. Sex identification in birds using two CHD genes. Proc R Soc Lond B Biol Sci. 263:1251–1256.
Groothuis TGG, Eising CM, Blount JD, Surai PF, Apanius V, Dijkstra C, Müller W. 2006. Multiple pathways of maternal effects in black-headed gull eggs: constraint and adaptive compensatory adjustment. J Evol Biol. 19:1304–1313.
Groothuis TGG, Müller W, von Engelhardt N, Carere C, Eising C. 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. Neurosci Biobehav Rev. 29:329–352.
Hall ME, Blount DJ, Forbes S, Royle NJ. 2010. Does oxidative stress mediate a trade-off between growth and self-maintenance in structured families? Funct Ecol. 24:365–373.
Halliwell B, Gutteridge J. 2007. Free radicals in biology and medicine. Oxford: Oxford University Press.
Hartley RC, Kennedy MW. 2004. Are carotenoids a red herring in sexual display? Trends Ecol Evol. 19:353–354.
Isaksson C, Andersson S. 2008. Oxidative stress does not influence carotenoid mobilization and plumage pigmentation. Proc R Soc Lond B Biol Sci. 275:309–314.
Isaksson C, Uller T, Andersson S. 2006. Parental effects on carotenoid-based plumage coloration in nestling great tits, *Parus major*. Behav Ecol Sociobiol. 60:556–562.
Jablonka E, Lamb MJ. 1995. Epigenetic inheritance and evolution: the Lamarckian dimension. Oxford: Oxford University Press.
Karadas F, Pappas AC, Surai PF, Speake BK. 2005. Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. Comp Biochem Physiol B. 141: 244–251.
Konjufca VK, Bottje WG, Bersi TK, Erf GF. 2004. Influence of dietary vitamin E on phagocytic function of macrophages in broilers. Poultry Sci. 83:1530–1534.
Koutsos EA, Clifford AJ, Calvert CC, Klasing KC. 2003. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). J Nutr. 133:1132–1138.
Koutos EA, Garcia Lopez JC, Klasing KC. 2006. Carotenoids from in ovo or dietary sources blunt systemic indices of the inflammatory response in growing chicks (*Gallus gallus domesticus*). J Nutr. 136: 1027–1031.
Koutos EA, Garcia Lopez JC, Klasing KC. 2007. Maternal and dietary carotenoids interactively affect cutaneous basophil responses in growing chickens (*Gallus gallus domesticus*). Comp Biochem Physiol B. 147:87–92.
Lessells CM, Boag PT. 1987. Unrepeatable repeatabilities: a common mistake. Auk. 104:116–121.
Littell RC, Milliken GA, Stroup WW, Wolfinger RS. 1996. SAS system for mixed models. Cary (NC): SAS Institute Inc.
Lozano GA. 1994. Carotenoids, parasites and sexual selection. Oikos. 70:309–311.
Martin LB II, Han P, Lewittes J, Kuhlman JR, Klasing KC, Wikelski M. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol. 20:290–299.
McGraw KJ, Adkins-Regan E, Parker RS. 2005. Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colourful songbird. Naturwissenschaften. 92: 375–380.
Møller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF. 2000. Carotenoid-dependant signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult Biol Rev. 11:137–159.
Monaghan P, Metcalfe NB, Torres R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett. 12:75–92.
Mousseau TA, Fox CW. 1998a. Maternal effects as adaptations. New York: Oxford University Press.
Mousseau TA, Fox CW. 1998b. The adaptive significance of maternal effects. Trends Ecol Evol. 13:403–407.
Nakagawa S. 2004. A farewell to Bonferroni: the problems of low statistical power and publication bias. Behav Ecol. 15:1044–1045.
Newbrey JL, Reed WL. 2009. Growth of yellow-headed blackbird *Xanthocephalus xanthocephalus* nestlings in relation to maternal body condition, egg mass, and yolk carotenoids concentrations. J Avian Biol. 40:419–429.
Nussey DH, Pemberton JM, Pilkington JG, Blount JD. 2009. Life history correlates of oxidative damage in a free-living mammal population. Funct Ecol. 23:809–817.
Olson VA, Owens IPF. 1998. Costly sexual signals: are carotenoids rare, risky or required? Trends Ecol Evol. 13:510–514.
Palozza P. 1998. Prooxidant actions of carotenoids in biological system. Nutr Rev. 56:257–265.
Pérez C, Lores M, Velando A. 2008. Availability of nonpigmentary antioxidant affects red coloration in gulls. Behav Ecol. 19: 967–973.
Porter NA, Caldwell SE, Mills KA. 1995. Mechanisms of free radical oxidation of unsaturated lipids. Lipids. 30:277–290.
Remeš V, Krist M, Bertacche V, Stradi R. 2007. Maternal carotenoid supplementation does not affect breeding performance in the Great Tit (*Parus major*). Funct Ecol. 21:776–783.
Romano M, Caprioli M, Ambrosini R, Rubolini D, Fasola M, Saino N. 2008. Maternal allocation strategies and differential effects of yolk carotenoids on the phenotype and viability of yellow-legged gull (*Larus michahellis*) chicks in relation to sex and laying order. J Evol Biol. 21:1626–1640.
Royle NJ, Surai PF, Hartley IR. 2001. Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? Behav Ecol. 12:381–385.
Royle NJ, Surai PF, Hartley IR. 2003. The effect of variation in dietary intake on maternal deposition of antioxidants in zebra finch eggs. Funct Ecol. 17:472–481.
Royle NJ, Surai PF, McCartney RJ, Speake BK. 1999. Parental investment and egg yolk lipid composition in gulls. Funct Ecol. 13:298–306.
Rubolini D, Romano M, Bonisoli-Alquati A, Caprioli M, Saino N. 2009. Within-clutch egg size asymmetry covaries with embryo sex in the yellow-legged gull *Larus michahellis*. Behav Ecol Sociobiol. 63: 1809–1819.
Rubolini D, Romano M, Bonisoli-Alquati A, Saino N. 2006. Early maternal, genetic and environmental components of antioxidant protection, morphology and immunity of yellow-legged gull (*Larus michahellis*) chicks. J Evol Biol. 19:1571–1584.
Rubolini D, Romano M, Martinelli R, Saino N. 2006. Effects of elevated yolk testosterone levels on survival, growth and immunity of male and female yellow-legged gull chicks. Behav Ecol Sociobiol. 59:344–352.
Saino N, Bertacche V, Bonisoli-Alquati A, Romano M, Rubolini D. 2008. Phenotypic correlates of yolk and plasma carotenoid concentration in yellow-legged gull chicks. Physiol Biochem Zool. 81: 211–225.
Saino N, Bertacche V, Ferrari RP, Martinelli R, Møller AP, Stradi R. 2002. Carotenoid concentration in barn swallow eggs is influenced by laying order, maternal infection and paternal ornamentation. Proc R Soc Lond B Biol Sci. 269:1729–1733.

Saino N, Calza S, Møller AP. 1997. Immunocompetence of nestling barn swallows in relation to brood size and paternal effort. J Anim Ecol. 66:827–836.
Saino N, Ferrari RP, Romano M, Martinelli R, Lacroix A, Gil D, Møller AP. 2006. Maternal allocation of androgens and antagonistic effects of yolk androgens on sons and daughters. Behav Ecol. 17:172–181.
Saino N, Ferrari RP, Romano M, Martinelli R, Møller AP. 2003. Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. Proc R Soc Lond B Biol Sci. 270:2485–2489.
Saino N, Martinelli R, Romano M. 2008. Ecological and phenological covariates of offspring sex ratio in barn swallows. Evol Ecol. 22: 659–674.
Saino N, Romano M, Caprioli M, Ambrosini R, Rubolini D, Fasola M. 2010. Sex allocation in yellow-legged gulls (*Larus michahellis*) depends on nutritional constraints on production of large last eggs. Proc R Soc Lond B Biol Sci. 277:1203–1208.
Saino N, Romano M, Caprioli M, Ambrosini R, Rubolini D, Fasola M. Forthcoming 2011. Hatching asynchorny and offspring sex: an experiment on maternal effects in the yellow-legged gull. Ethol Ecol Evol.
von Schantz T, Bensch S, Grahn M, Hasselquist D, Wittzell H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc Lond B Biol Sci. 266:1–12.
Schwabl H. 1996. Maternal testosterone in the egg enhances postnatal growth. Comp Biochem Physiol C. 114:271–276.
Surai PF. 2002. Natural antioxidants in avian nutrition and reproduction. Nottingham (UK): Nottingham University Press.
Surai PF, McDevitt RM, Speake BK, Sparks NHC. 1999. Carotenoid distribution in tissues of the laying hen depending on their dietary supplementation. Proc Nutr Soc. 58:30A.
Surai PF, Noble RC, Speake BK. 1996. Tissue-specific differences in antioxidants distribution and susceptibility to lipid peroxidation during development of the chick embryo. BBA Mol Cell Biol L. 1304:1–10.
Surai PF, Speake BK, Sparks NHC. 2001. Carotenoids in avian nutrition and embryonic development. 2. Antioxidant properties and discrimination in embryonic tissues. J Poultry Sci. 38:117–145.
Surai AP, Surai PF, Steinberg W, Wakeman WG, Speake BK, Sparks NHC. 2003. Effect of canthaxanthin content of the maternal diet on the antioxidant system of the developing chick. Br Poult Sci. 44: 612–619.
Tella JL, Lemus JA, Carrete M, Blanco G. 2008. The PHA test reflects acquired T-cell mediated immunocompetence in birds. Plos One. 3:e3295.
Tella JL, Scheuerlein A, Ricklefs RE. 2002. Is cell-mediated immunity related to the evolution of life-history strategies in birds? Proc R Soc Lond B Biol Sci. 269:1059–1066.
Tschirren B, Fitze PS, Richner H. 2003. Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. J Anim Ecol. 72:839–845.
Tschirren B, Saladin V, Fitze PS, Schwabl H, Richner H. 2005. Maternal yolk testosterone does not modulate parasite susceptibility or immune function in great tit nestlings. J Anim Ecol. 74:675–682.
Verboven N, Evans NP, D'Alba L, Nager RG, Blount JD, Surai PF, Monaghan P. 2005. Intra-specific interactions influence egg composition in the lesser black-backed gull (*Larus fuscus*). Behav Ecol Sociobiol. 57:357–365.
Wade MJ. 1998. The evolutionary genetics of maternal effects. In: Mousseau TA, Fox CW, editors. Maternal effects as adaptations. New York: Oxford University Press. p. 5–21.
Wittingham MJ, Bradbury RB, Stephens PA, Freckleton RP. 2006. Why do we still use stepwise modelling in ecology and behaviour? J Anim Ecol. 75:1182–1189.
Wolf JB, Brodie ED III, Cheverud JM, Moore AJ, Wade MJ. 1998. Evolutionary consequences of indirect genetics effects. Trends Ecol Evol. 13:64–69.
Zera AJ, Harshman LG. 2001. The physiology of life history trade-offs in animals. Annu Rev Ecol Evol Syst. 32:95–126.